



## The Association between erythropoietin, vitamin D3 and parathormone levels and kidney lesions in buffaloes (*Bubalus bubalis*)

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### ABSTRACT

Renal disorders in livestock may appear subclinically, leading to reduced production performance without any clinical signs, and causing significant economic losses. In this research, blood and kidney tissue samples were obtained from 102 buffaloes (males and females) slaughtered at Ahvaz industrial slaughterhouse and sent to the laboratory for biochemical and histopathological analyses. Having used the conventional and specific staining methods along with the microscopic examination of the histopathological sections of the kidney tissue samples from buffaloes, 41 samples with no lesion were selected as control and 61 with renal lesions were considered as the lesion group. The samples with renal lesions were then divided into 4 subgroups including acute tubulointerstitial nephritis, chronic tubulointerstitial nephritis, urinary tubular inflammation, and congestion. Serum biochemical tests including parathyroid hormone, erythropoietin, and Vit D3 were measured in blood samples using commercial ELISA kits. After the statistical analysis of the data, the results of Vit D3 in the studied groups did not show any significant differences. However, the levels of erythropoietin and parathormone in buffaloes with renal lesions were significantly lower and higher than those in the control animals, respectively. The results also showed that most of the kidney lesions in the studied buffaloes led to a decrease in the production of erythropoietin hormone following the occurrence of such lesions and their functional effects on the infected kidneys. Besides, the effects of erythropoietin deficiency were reflected in the hemogram, and its resulting anemia was deemed to affect overall body health. As the findings showed, no significant differences were observed between male and female buffaloes in terms of the abundance of renal lesions and laboratory findings.

### Keywords

*Bubalus bubalis, Kidney lesions, Erythropoietin, Vitamin D3, Parathormone*

### Abbreviations

EPO: Erythropoietin  
Vit D3: Vitamin D3  
PTH: Parathormone  
ATN: Acute tubulointerstitial nephritis  
CTN: Chronic tubulointerstitial nephritis  
UTI: Urinary tubular inflammation  
CKD: Chronic kidney disease

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### Introduction

Water buffalo (*Bubalus bubalis*) is an important livestock resource in many countries of Asia, including Iran, the Mediterranean region, and Latin America. It plays an important role in providing milk, meat, and draught power in agricultural systems, particularly in the developing countries. This productive, adoptive and multipurpose domestic animal species has thus received significant attention in national and international livestock arena in recent years. In addition to supplying the motive power for agriculture allied activities and transport, there is indeed a higher need for raising and successful maintenance of buffalo milk, milk products and meat production in the agriculture-based countries (1).

The breeding of buffalo as a source of milk, meat and labor in tropical regions of Iran and South Asian countries are thus of special economic importance (2). West and East Azarbaijan provinces, Khuzestan, Ardebil, Gilan, Mazandaran, Golestan and Lorestan are considered to be the breeding centers of buffaloes in Iran. According to the statistics provided by the Statistical Centre of Iran in 2017, the number of Iranian buffaloes is estimated to be 127 thousand, showing a decreasing trend in number of buffaloes than in previous years. In effect, due to the lack of proper breeding management, the rise in demand for buffalo meat and the lack of replacement for the slaughtered animals, have resulted in a decline in the population of buffaloes in Iran.

Though its natural habitat consists of hot and humid regions that are very favorable to microorganism and parasite proliferation, *Bubalus bubalis* is generally a healthy animal (3). However, like many other animal species, *Bubalus bubalis* is susceptible to most diseases and parasites that afflict cattle, though the effects of such diseases on the buffalo and its productivity are sometimes less evident. Among others, renal diseases are not uncommon in food animals and information from slaughterhouses can be considered a good source of data for the evaluation and monitoring of renal diseases in livestock (4).

Among body organs and tissues, kidney plays an important role in maintaining the health and adjustment of the internal environment of the body, and any damage to it may cause changes in blood biochemical parameters. Infectious agents, toxins, impacts, immune responses, kidney hypoxia, tumors, and the formation of urinary stones are among the causative agents of the kidney. Thus, awareness of such changes in kidney before the advent of clinical symptoms is the first essential step in improving the methods of prevention and management in livestock farms. In addition to economic losses, diseases affecting buffa-

loes might constitute an epidemiologic and zoonotic threat to humans. As such, problems concerning meat hygiene and possible health risks to consumers should be well documented during both ante-mortem and post-mortem examinations.

Different affections of kidney include developmental anomalies, growth, metabolic and circulatory disturbances, infarction and necrosis, diseases of glomeruli (viral glomerulonephritis, embolic nephritis, immune-mediated glomerulonephritis, immune-complex glomerulonephritis, glomerulosclerosis and glomerulolipidosis), tubulo-interstitial diseases (interstitial nephritis, granulomatous nephritis, and pyelonephritis), chronic renal diseases, urolithiasis and various neoplasms (5). However, there is little information on the renal pathological changes in buffaloes compared to those in other animal species (6, 7).

Erythropoietin (EPO), as a glycoprotein hormone produced primarily by the kidney, is a principal growth factor regulating the red blood cell production (8). Kidney is indeed the major EPO producing organ in adult life, while the liver produces only 10-15% of the total amount of EPO produced in the body (9).

It is also established that vitamin D is not simply a nutrient required for normal skeletal growth and development; rather it serves as a precursor to an intricate endocrine mechanism that maintains calcium and phosphorus concentrations in blood. More recently, vitamin D has been shown to have multiple physiological roles, including the control of cellular differentiation and proliferation as well as the activation of innate immune defenses. The new-found roles of vitamin D, along with its critical role in the calcium and phosphorus homeostasis, highlight the need to examine current practices for vitamin D supplementation in the dairy industry and question whether current recommendations are adequate for dairy cattle (10).

In turn, parathyroid hormone, as a single-chain polypeptide made up of 84 amino acids secreted by the chief cells in the parathyroid gland, augments the absorption of calcium from the intestines and simultaneously conditions its re-absorption from the skeleton. Also, parathormone (PTH) is the principal hormone involved in the fine regulation of the calcium homeostasis. The hypercalcemic biological action of PTH is indeed by its direct influence on the target cells in bones (osteoclasts and osteocytes) and kidney (proximal and distal tubules) along with its indirect action on the duodenum (11, 12).

Kidney lesions, in addition to having a direct impact on regulating the body's homeostasis, disrupt the production and metabolism of some hormones that directly affect the health of the kidney tissue. There-

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fore, this research was aimed to study different types of histopathological disorders of kidney, the changes in EPO, PTH, and Vit D3 along with the correlation between the hormonal changes and histopathologic lesions. To those ends, blood and kidney samples were collected from buffaloes slaughtered at Ahvaz industrial slaughterhouse so as to investigate the pathomorphological and hormonal alterations in the samples.

**Table 1.**

Specimens from studied buffaloes according to gender and renal lesions.

Buffaloes	Male	Female	Total
Normal	32	9	41
Kidney lesion	56	5	61
Total	88	14	102

**Table 2.**

Changes in the Mean  $\pm$  SE values of EPO, parathyroid hormone and Vit D3 in buffaloes according to renal lesions.

Group	Number	%	EPO	PTH	Vit D3
Healthy	41	40.20	49.32 $\pm$ 4.089 <sup>bcd</sup>	21.34 $\pm$ 4.520 <sup>c</sup>	69.44 $\pm$ 6.399
ATN	18	17.65	31.89 $\pm$ 2.747 <sup>a</sup>	22.44 $\pm$ 3.601 <sup>c</sup>	63.06 $\pm$ 6.503
CTN	23	22.55	33.50 $\pm$ 2.846 <sup>a</sup>	31.50 $\pm$ 3.417 <sup>abdef</sup>	64.25 $\pm$ 4.642
UTI	13	12.74	34.69 $\pm$ 2.041 <sup>a</sup>	22.69 $\pm$ 4.385 <sup>c</sup>	65.15 $\pm$ 5.581
Congestion	7	6.86	36.43 $\pm$ 3.036 <sup>a</sup>	22.57 $\pm$ 3.359 <sup>c</sup>	67.86 $\pm$ 4.340
Total	102	100	37.28 $\pm$ 11.398	22.89 $\pm$ 4.693 <sup>c</sup>	64.85 $\pm$ 6.97

Different lower case letters (a-f) demonstrate significant differences between groups ( $p < 0.05$ )

### Results

#### Histopathological findings

In this study, 102 kidney samples were obtained from apparently healthy buffaloes (88 males and 14 females) slaughtered at Ahvaz industrial slaughterhouse. Among them, 61 kidney samples had lesions and 41 others were healthy. From kidney samples with lesions, fifteen cases (14.7%) had gross lesions such as enlargement of kidney, severe congestion and hemorrhages. All kidney samples were then examined in the laboratory so as to identify the histopathological renal changes in buffaloes. The prevalence of histopathological renal changes including acute tubulointerstitial nephritis (ATN), chronic tubulointerstitial nephritis (CTN), urinary tubular inflammation (UTI) and congestion is presented in Table 2.

#### Analysis of hormones

The *t*-test analysis showed that the presence or absence of any renal injury was significantly correlated with the serum EPO levels ( $p < 0.001$ ), and it was significantly lower in the groups with renal lesions than

in the healthy one. As for vitamin D, there was no significant difference between healthy and kidney lesion groups. However, there was a significant elevation in PTH in CTN group compared to the healthy and other groups with different kidney lesions. This was likely related to the secondary renal hyperparathyroidism.

#### Statistical analysis

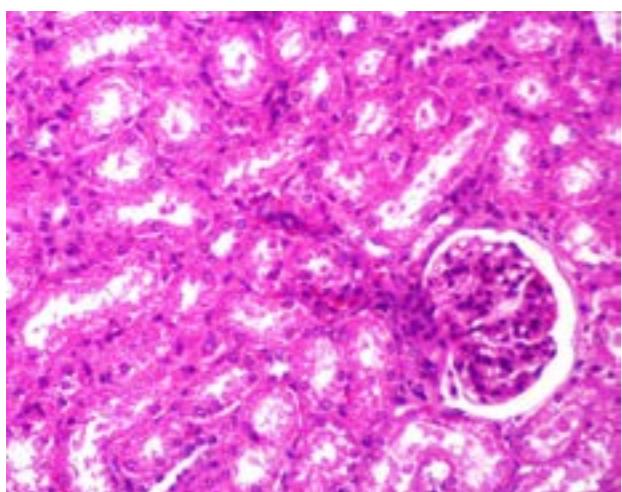
The results of statistical analysis for the groups with different lesions showed that EPO level in healthy group was significantly different from that in all other groups. It was also found that EPO level in the healthy group was significantly higher than that in other groups. Besides, the results showed that there were no significant differences between different groups with lesions in EPO levels. The two-way analysis of variance with two factors of gender and kidney lesions showed that renal injury alone was effective in the EPO levels ( $p < 0.001$ ), while gender had no significant effect on the EPO levels. In the case of Vit D3, neither injury nor gender had a significant effect on the EPO values ( $p > 0.05$ ).

**Discussion**

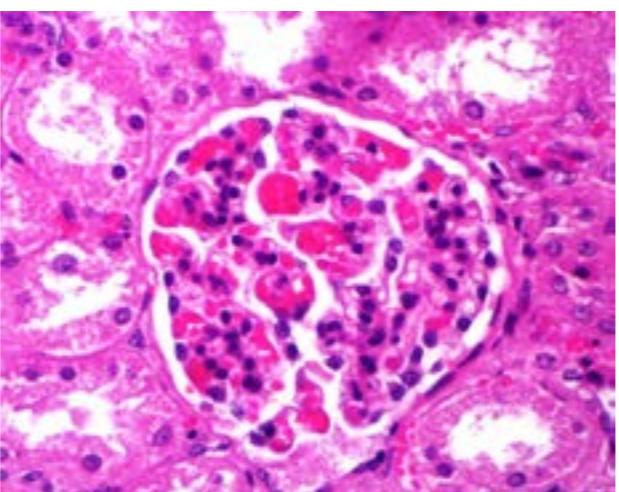
Little is known about the pathological kidney lesions in buffaloes in comparison to other animal species. Although some cases of kidney lesions in other animal species slaughtered at Ahvaz slaughterhouse have been investigated in some research studies, no study has been already conducted on buffalo kidney lesions in Khuzestan province. Thus, this research investigated the association between histopathological kidney lesions in slaughtered buffaloes and any possible changes in important indicators such as EPO, parathyroid hormone and Vit D3. As the findings showed, interstitial nephritis was considered as the most common pathological finding in this study. Although interstitial nephritis, either diffuse or focal, is most frequent in post-slaughter inspection in some species, it is rarely detectable in clinical examinations. However, chronic focal interstitial nephritis in kidney, known as a white spot, is a common finding in apparently healthy animals in post-slaughter inspection.

The results showed that from the 102 samples examined, 41 had no renal lesions (40.2%, Fig. 1). Also, most of the lesions were interstitial nephritis, comprising 41 samples (18 acute and 23 chronic samples) or 40.2% of the total cases (Figs. 3, 4). Of these, chronic interstitial nephritis had the highest number of lesions (22.5%), including connective tissue replacement in the interstitial spaces, especially around the glomerular network, infiltrated by mononuclear cells such as macrophages and lymphocytes. Besides, the EPO level was significantly decreased in the groups with chronic interstitial nephritis compared to the healthy group; however, no significant difference was observed in PTH and Vit D3 values between the groups.

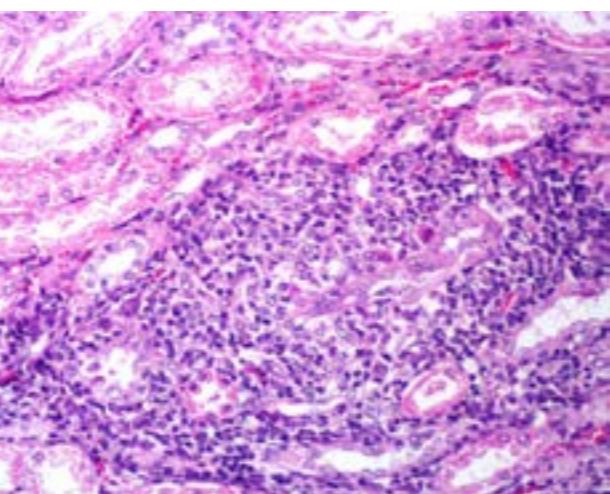
Acute interstitial nephritis was, in turn, found



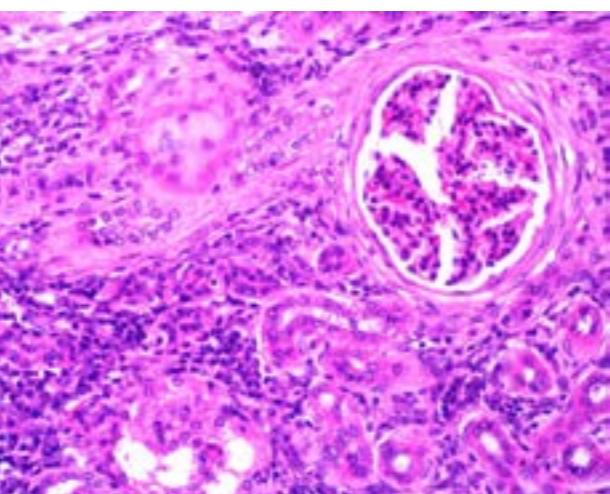
**Figure 1.**  
The structure of the kidney tissue including the glomerular corpuscle and the urinary tubules is normally observed. H & E staining. Magnification: 100x



**Figure 2.**  
Glomerular congestion and the decreased glomerular urinary pole space. H & E staining. Magnification: 400x



**Figure 3.**  
The presence of mononuclear cells in the interstitial space, necrosis of the urinary tubules and the congestion of the affected areas. H & E staining. Magnification: 100x

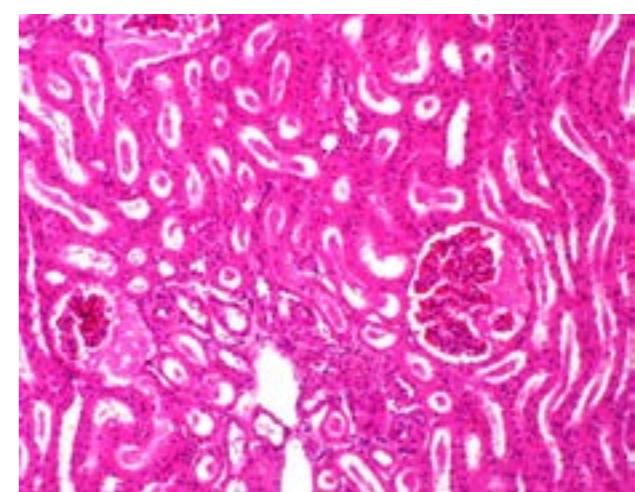


**Figure 4.**  
The aggregation of fibroblasts around glomeruli, infiltration of mononuclear cells into interstitial tissue, the accumulation of connective tissue and the collagen fibers in the interstitial space without the presence of any congestion. H & E staining. Magnification: 200x

rix (ECM) proteins in the renal interstitium, a finding known as interstitial fibrosis (14). The results of Vit D3 concentration showed that all samples examined were within a normal value range, and there was no significant difference between the healthy and lesion groups in this regard. However, a statistically significant negative correlation was found between the levels of EPO and PTH in the CTN group. Indeed, the secondary hyperparathyroidism, as a common complication of CKD, resulted from the interaction of several different factors initiated by the loss of kidney tissue and the inability to excrete the daily load of phosphates, causing an increase in its serum levels. In effect, hyperphosphatemia stimulates the posterior liberation of fibroblastic growth factor 23 (FGF23) by osteocytes, which

inhibits proteins NaPiIIa and NaPiIIC in the proximal convoluted tubule generating phosphaturia. It also inhibits the activity of the 1 $\alpha$ -hydroxylase renal enzyme, diminishing the synthesis of active vitamin D (1, 25(OH)2D), leading to a reduction in the intestinal absorption of calcium and phosphorus, and serum levels of phosphorus. The final outcome of lowering phosphorus is an increase in the levels of FGF23 and hypocalcaemia, the latter causes an increase in the synthesis and liberation of parathyroid hormone (15, 16).

In Restrepo and Aguirre (2016) study on the level of vitamin D in patients with CKD stages, 21.1% of the patients had normal vitamin D levels, 70.1% were within the insufficient range, while 8.8% were in deficit. Moreover, a negative correlation was found between the levels of vitamin 25(OH)D and the values of PTH. They also found low levels of vitamin 25(OH)D in patients with CKD, leading to the appearance of secondary hyperparathyroidism. The active vitamin D or calcitriol has indeed important functions in patients with CKD as it promotes the intestinal absorption of calcium and phosphorus, increases the distal tubular absorption of calcium in the kidney and exerts negative feedback on the parathyroid gland, lowering the synthesis and secretion of the parathyroid hormone (PTH). Thus, a sufficient supply of native vitamin D (25(OH)D) in the form of ergocalciferol (vitamin D2) or cholecalciferol (vitamin D3) is required for its synthesis in the kidney's proximal convoluted tubule. As such, a progressive reduction in the levels of active vitamin D has been observed in patients with CKD proportional to the decrease of their glomerular filtration rate. It has been assumed that this hap-



**Figure 5.**  
Protein leakage, hyaline casts, and the necrosis of the urinary tubule cells are observed. H & E staining. Magnification: 100x

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pens due to a smaller amount of renal mass and the decrease in the number of proximal tubular cells that absorb the filtered native vitamin D (25(OH) D) to then be hydroxylated to its active form by the 1 $\alpha$ -hydroxylase (17).

Nikodimopoulou and Liakos (2011) reported that the secondary hyperparathyroidism (SHPT), as a common disorder in patients with CKD, is characterized by excessive serum parathyroid hormone (PTH) levels, parathyroid hyperplasia and an imbalance in the calcium and phosphorus metabolism. In turn, the SHPT develops early in the course of CKD and becomes more prominent as the kidney function declines (18). Interstitial nephritis is indeed caused by different infective agents including bacterial, viral and protozoal agents such as *Escherichia coli*, sheep pox virus, Adenovirus and *Leptospira* (19).

To determine the prevalence and type of urinary bladder and renal lesions in the slaughtered buffaloes at Ahvaz industrial slaughterhouse, Mohamadian et al. (2016) randomly selected a total of 353 buffaloes for gross histopathological studies. The histopathologic findings showed that 128 (36.3%) of the examined animals had renal lesions including interstitial nephritis (27.2%), acute tubular necrosis (ATN) (3.1%), hydronephrosis (2.5%), glomerular capillary hemorrhage (1.7%), pyelonephritis (1.4%), glomerulonephritis (0.3%). Besides, interstitial nephritis was found to be the major observed lesion (75%) and also the most common histopathological lesions of the kidney identified in buffaloes. As the statistical analysis showed, gender and age were not correlated with the renal lesions (20).

In another research, Nikvand, et al. (2014) showed that the isolation of bacteria from urine was not related to cystitis and pyelonephritis. Indeed, some cases of chronic cystitis were not able to isolate bacteria from urine and for causing pyelonephritis they need predisposing factors which caused urine retention (21).

According to histopathological findings of Baghban and Yaripour (2016), interstitial nephritis was the most abundant condition observed in 69 cases (71.13%) of sheep kidneys and 68 cases (72.34%) of goat kidneys. The other renal lesions observed in sheep included purulent abscess in 15 cases (15.47%), pyelonephritis in 6 cases (6.18%), nephrosis in 5 cases (5.16%) and hydatid cyst in 2 cases (2.06%). In goats, the renal lesions were purulent abscess in 10 cases (10.64%), pyelonephritis in 4 cases (4.25%), nephrosis in 4 cases (4.25%), amyloidosis in 2 cases (2.13%), hydatid cyst in 3 cases (3.19%), infarction in 2 cases (2.25%) and congestion in 1 case (1.07%). The statistical analysis showed a significant difference ( $p < 0.05$ ) in relation to the lesions observed in the condemned kidneys between the two sexes. The results also

showed that interstitial nephritis followed by purulent abscesses and pyelonephritis was the most abundant lesion in the condemned kidneys of sheep and goats (22).

Examining sheep from different breeds in Turkey, Hatipoglu et al. (2001) found renal abnormalities macroscopically and microscopically in 316 (3.13%) head of sheep. The most prevalent renal lesions in sheep was interstitial nephritis in 203 (62.24%) head of sheep (23).

In another study, Ansari-Lari (2007) determined gross abnormality changes in the condemned kidneys of sheep and goats to be 1.5-3% in the Shiraz slaughterhouse. Also, Ansari-Lari reported the abundance of kidney condemnation in sheep and goats to be 1.1 and 2.3%, respectively. Moreover, nephritis was identified as the most important reason of kidney condemnation in sheep and goats with 25% and 16% abundance, respectively (4). In another study, Mathur and Dadhich (2005) reported that from 1284 head of sheep examined, 223 head of sheep with apparent macroscopic lesions showed interstitial nephritis in 45 (20.17%) samples (24).

Investigating bovine kidney diseases in Shahrekord district, Kojouri et al. (2008) showed that the prevalence of renal diseases in Shahrekord slaughterhouse was approximately 7.9%. The relative frequency of disorders was reported as interstitial nephritis (34.18%), cyst (27.85%), hydatid cyst (11.4%), glomerulonephritis (3.8%), acute tubular necrosis (5.06%), fibrosis (3.8%), hydronephrosis (3.8%), abscess (2.53%), hemorrhage and congestion (3.8%), renal dysplasia (1.26%), infarction (1.26%) and hyaline cast (1.26%) (25).

Using the slaughterhouse data in Dublin, Monaghan and Hannan (1983) reported that 4.2% of 4166 cattle slaughtered had kidneys that were rejected due to gross abnormalities. The rejection rate was 7.7%, 1.7%, 2.2% and 28% for cows, bullocks, heifers, and bulls, respectively. Besides, the focal interstitial nephritis was found to be the most frequent renal lesion (60.1%) in the slaughtered cattle (26).

Similarly, investigating the prevalence and types of renal lesions, Nourmohammadzadeh et al. (2010) reported that 35 (8.6%) of the examined animals had renal lesions including interstitial nephritis (85.7%), cyst (11.4%), glomerulonephritis (5.7%), ATN (5.75%), pyelonephritis (2.85%), amyloidosis (2.85%), leukosis (2.85%), hydronephrosis (2.85%), and unilateral renal aplasia (2.85%). Moreover, the prevalence of renal lesions in female and male cattle was 8.5% and 9.4%, respectively, which was not statistically significant. Likewise, the prevalence of renal lesions in cows and heifers was found to be 10% and 2.8%, respectively, which was statistically significant (27).

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In another study, aiming to assess the prevalence of renal lesions in the slaughtered cattle in the Shiraz slaughterhouse, Taghadosi, et al. (2016) reported the correlation between the rejected kidneys and infection with *Leptospira* using the nested PCR-restriction fragment length polymorphism (RFLP) techniques. The findings showed that out of 1000 inspected animals, 205 (20.5 %) had renal lesions. Among them, chronic nephritis (7.5%), white-spotted kidney (7.3 %), and petechial hemorrhage (3.5 %) were the most prevalent forms of the lesions. Moreover, a direct correlation between increasing the age and a significant increase in the rate of lesions was also observed (29).

### Conclusion

As the findings showed renal disorders in livestock may appear subclinically leading to their reduced production performance without any clinical signs. Thus, further studies are required to determine the other aspects of renal subclinical diseases in the cattle slaughtered. Moreover, the changes in the level of kidney function-related hormones such as EPO, PTH, and vitamin D were found likely to affect the metabolism of many organs, causing significant economic losses by reducing the growth and production in the meat and dairy industry. The results of this study could thus help veterinary clinicians and other stakeholders in the field of buffalo breeding improve their knowledge about the role of healthy kidney in the maintenance of buffalo healthy conditions.

### Material and methods

Having identified the specifications of buffaloes, 5 ml of blood samples were taken from each animal in clot tubes immediately after the slaughter at Ahvaz industrial slaughterhouse. The blood samples were centrifuged after being transferred to the laboratory at 2500 rpm for 10 minutes. Then, the serum was separated and stored in a freezer at -70 °C until the tests were carried out. The ELISA method was used to measure the levels of EPO, PTH and serum Vit D3. The EPO ELISA kit (Mybiosource, cat no: MBS907898; USA) assay has high sensitivity and excellent specificity for detection of bovine EPO. No significant cross-reactivity or interference between bovine EPO and analogues was observed.

Bovine parathyroid hormone (PTH) was, in turn, measured by the ELISA Kit (Abbkine cat: KTE10155; USA). The PTHELISA Kit employs a two-site sandwich ELISA to quantitate PTH in the samples. Vitamin D ELISA kit (Mybiosource, cat no: MBS744057; USA) assay has high sensitivity and excellent specificity for the detection of Vitamin D. No significant cross-reactivity or interference between Vitamin D and analogues was observed.

After the slaughter was done, kidneys were isolated from the buffalo carcasses to examine their appearance and record any probable macroscopic lesions. In case of any macroscopic lesion or abnormality, a proper sample was taken from the lesion site, while in the absence of any macroscopic lesion, the sample was taken from one of the lobes near the umbilical region of the kidney and transferred to the pathology lab in 10% formalin. The tissue samples were then placed in a 10% formalin buffer container (10 times the sample volume) and 24 hours later, formalin was replaced. Af-

ter a week, each sample was made up of sections of 2 to 3 mm in diameter and placed in special containers with specified numbers. If the specimen had a macroscopic lesion, it was taken from the same site. Afterwards, the baskets were placed under running water for 4 hours and then transferred to the Autotechnicon (Leica, Germany) tissue processor. The process of tissue passage involving dehydration, clearing and impregnation was carried out on the tissue processor. After 18 hours, the specimens were extracted from the device and paraffin blocks were prepared. The blocks stored in the laboratory temperature and also in the refrigerator for one day were prepared using a 5-micrometer section of the microtome. After placing the tissue sections on the slide, paraffin was melted in a hot air oven at 50-60 degree centigrade depending upon the melting point. Then, the samples stained with the hematoxylin-eosin method were used for histopathological evaluation. If necessary, the Periodic acid-Schiff (PAS) staining method was also used. After careful examination of the prepared slides, healthy kidney specimens were separated and the lesions samples were divided into five groups according to the type and severity of the lesions including ATN, CTN, chronic multifocal tubulointerstitial nephritis, UTI and congestion. Then, different types of histopathological disorders of kidney, the changes in EPO, PTH, and vitamin D values along with the correlation between the hormonal changes and histopathologic lesions were studied statistically using SPSS-22 software and the related tests.

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### Author Contributions

Research design: M.R.J and M.R. H.H. Clinical sampling: Z.S. Pathological studies: B.M. Measurement of hormones: M.T.J.

### Conflict of Interest

The authors declare that they have no conflict of interest

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